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Chemical Composition of Leaf Essential Oil of *Juniperus phoenicea* and Evaluation of its Antibacterial Activity

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ABSTRACT

In this work, the chemical composition and antimicrobial activity of essential oils obtained from *Juniperus phoenicea* were determined. *Juniperus* species from the Cupressaceae family are widely distributed in Morocco. In this study, the essential oils of *J. phoenicea* collected from Atlas median in the region of Boulmane (Morocco) were obtained by hydro-distillation of the aerial parts and analysed by gas chromatography equipped with flame ionisation detector (GC-FID) and gas chromatography coupled to a mass spectrometry system (GC/MS) for their chemical composition. Their antibacterial activity was studied *in vitro* on seven bacterial strains: *Escherichia coli, Staphylococcus aureus, Staph. intermedius, Klebsiella pneumonia, Pseudomonas aeruginosa, Bacillus subtilis* and *Streptococcus mutans*. Twenty tree compounds were identified in leaves oil representing 81.87% of the total oil composition. The yield of essential oil of *J. phoenicea* was 1.62% and the major compound in aerial parts was α-pinene (49.15%) followed by α-phyllandrene (7.39%), mycene (5.24%), β-pinene (3.58%), linalool (2.54%), piperitone (1.56%), γ-terpinene (1.28%), Trans-pinocarveole (1.23%) ρ-cymene (1.10%), α terpineol (1.02%) and γ-cardinene (1.01%). The bacterial strains tested were found to be sensitive to essential oils studied and showed a very effective bactericidal activity with minimum inhibitory concentrations (MIC) ranging from 0.02 to 0.40 mg per mL.

Key Words: *Juniperus phoenicea;* Chemical composition; α-pinene; Antibacterial activity

INTRODUCTION

Juniperus phoenicea. (Fam. Cupressaceae) is the species found in Morocco. It extends to Egypt (Elsawi *et al.*, 2007) and Central Arabia (Boulos, 1999). The leaf essential oil of *J. phoenicea* has been reported in varying details from Saudi Arabia (Dawidar *et al.*, 1991), France (Tabacik & LaPorte, 1971; Tabacik & Poison, 1971) and from Greece and Spain. Also, there are some reports on the analysis of fruit essential oils (Delitala, 1980; De Pascual *et al.*, 1981; Ramic & Murko, 1983).

Medicinal plants have been used for centuries as remedies for human diseases, because they contain chemical components of therapeutic value (Nostro *et al.*, 2000). According to the World Health Organization (WHO) in 2008, more than 80% of the world's population relies on traditional medicine for their primary healthcare needs (Pierangeli *et al.*, 2009). Essential oils are valuable natural products used as raw materials in many fields, including perfumes, cosmetics, aromatherapy, phototherapy, spices and nutrition (Buchbauer, 2000). Also the Essential oils are used in traditional medicine for their antiseptic action are constituted 1% of plant secondary metabolites and are mainly represented by terpenoids, phenypropanoids or benzenoids, fatty acid derivatives and amino-acid derivatives (Dudareva *et al.,* 2006).

J. phoenicea, is small tree that is native to the northern lands bordering the Mediterranean Sea from Portugal to Israel. It is also native to North Africa in Algeries, Morocco and Canary Islands (Gaussen, 1968). The essential oils, which were utilised centuries ago in cosmetics usually show interesting biological features. The oils also help increase the flow of digestive fluids, improve digestion and eliminate gas and stomach cramping (Uphof, 1968). *J. phoenicea* has been used for centuries as a steam inhalant for bronchitis and to control arthritis. The oil is also irritating to microbes, so much so that it kills many of them (Watt *et al*., 1962; Stassi *et al.*, 1996).

In the light of this work we have determined, the chemical composition and the antibacterial activity of

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essential oils of leaves of *J. phoenicea* collected in the region of Boulmane in Morocco, where the inhabitants frequently use these plants in traditional medicine.

MATERIALS AND METHODS

Vegetal material and essential oil extraction: The visible parts of *Juniperus phoenicea* have been collected during March 2009 in the region of Boulmane, 90 km in the south east of Fez city (latitude: 25° 31 '11" longitude: 5° 22' 21"; altitude: 2100 m); the climate is semi-humid with strong continental influence having an annual average temperature of 20°C, east of Fez city. The collected leaves were then dried in the open air for fifteen days. The plants were then isolated from the other specimen and conserved for extraction.

The essential oils were extracted by hydro-distillation using an apparatus of Clevenger type. The extraction took 3 h for mixing 250 g of plants in 1600 mL of distilled water. After filtration the solvent is eliminated by pressure distillation reduced in rotary evaporator at 35°C and pure oil was stored at 4°C in obscurity till the beginning of analysis. The essential oils yield is demonstrated by the oil quality (in mL) obtained for 100 g of dried leaves.

Gas chromatography analysis (GC-FID & GC/MS): The chemical composition of leaf oil from *J. phoenicea* in Morocco was determined by GC-FID and GC-MS using a CP-SIL5HP fused silica column.

The GC (Trace GC ULTRA, Thermo Fischer) analysis equipped with flame ionisation detector (GC-FID), Varian capillary column (CP-Sil 5 HP, 60 m length, 0.32 mm of diameter & Film thickness 0.25 µm). The column temperature was programmed from 40 to 280°C for 5°C/min. The temperature of the injector was fixed to 250°C and the one of the detector (FID) to 260°C. The debit of gas vector (nitrogen) was fixed to 1 mL/min. The volume of injected specimen was 0.5 µL of diluted oil in hexane solution (10%). The percentage of each constituent in the oil was determined by area peaks.

The identification of different chemical constituents was done by gas phase chromatography (Ultra GC Trace) coupled with mass spectrometry (PolarisQ, Thermo Fischer) (GC/MS). The utilised column was; Varian capillary column (CP-Sil 5 HP; 60 m length, 0.32 mm of diameter & Film thickness 0.25 µm). The column temperature was programmed from 50 to 280°C for 3°C/min. The temperature of the injector was fixed to 240°C and the one of the detector to 200°C. Electrons impact: 70ev. The debit of gas vector (Helium) was fixed to 1.5 mL/min. The volume of injected specimen was of 1 µL of diluted oil in hexane solution (10%). The constituents of essential oils were identified in comparison with their Kovats Index, calculated in relation to the retention time of a series of lineary alkanes (C_4- C_{28}) with those of reference products and in comparison with their covets index with those of the chemical components gathered by Adams (2001) and in comparison with their spectres of mass with those gathered in a library of (NIST-MS) type.

Antibacterial activity: In recent years due to an upsurge in antibiotic-resistant infections, the search for new prototype drugs to combat infections is an absolute necessity and in this regard plant essential oils may offer great potential and hope. Volatile compounds from plants, especially essential oils have antimicrobial, fungicidal and insecticidal activities (Wilson *et al.*, 1997; Malika *et al.,* 2004; Reinhard *et al.*, 2004). These products have frequently been reported to be antimicrobial agents (Martinez, 1973; Franchomme, 1981; Benjilali *et al.*, 1984; Tantaoui- Elaraki *et al.*, 1992; Panizzi *et al.,* 1993; Remmal, 1994; Mouhssen, 2004; Anwar *et al*., 2009). The volatile essential oils released from leaves, flowers and fruits into the atmosphere and from roots into the soil defend herbivores and pathogens (Dudareva *et al.*, 2006).

The selected essential oils were screened against four: *Escherichia coli, Staphylococcus aureus*, *Staph. intermedius, Klebsiella pneumonia, Pseudomonas aeruginosa, Bacillus subtilis* and *Streptococcus mutans.*

The minimal inhibition concentration (MIC) values were evaluated according to published procedures (Koneman *et al.*, 1997; Iscan *et al.*, 2002; Guven *et al.*, 2005). The minimal inhibitory concentration (MIC) was determined only with micro-organisms that displayed inhibitory zones. MIC was determined by dilution of the essential oils in dimethyl sulfoxide (DMSO) and pipetting 0.01 mL of each dilution into a filter paper disc. Dilutions of the oils within a concentration range of 0.02- 0.40 mg/mL were also carried out. MIC was defined as the lowest concentration that inhibited the visible bacterial growth (NCCLS, 2005). A negative control was also included in the test using a filter paper disc saturated with DMSO to check possible activity of this solvent against the bacteria assayed. The experiments were repeated at least twice. Antibacterial activity of *J. phoenicea* oil was examined using different bacterial species. In addition, chemical composition of volatile compounds, were also determined.

RESULTS AND DISCUSSION

Chemical composition of the essential oil: The retention time and chemical composition of essential oils of *J. phoenicea* are presented in Fig. 1 and Table I.

The constituents of *J. phoenicea* from Morocco are listed in order of their elution on the CP-Sil 5 HP column (Fig. 1). In total twenty tree volatile compounds, representing 81.87% of the total composition, were identified in the leaves oils (Table I). Monoterpene hydrocarbons (70.19%) were found to be the major group of compounds, the main one being α pinene (49.15%) followed by α -phellandrene (7.39%). The most abundant components found in the leaf oil were α-pinene (49.15%) followed by α-phellandrene (7.39%), mycene (5.24%), β-pinene (3.58%), linalool (2.54%), piperitone (1.56%), γ-terpinene (1.28%), Trans-pinocarveol (1.23%) ρ-cymene (1.10%), α terpineol (1.02%) and γ-cadinene (1.01%). The essential oils yield of *J. phoenicea* collected from region of

Table I: Chemical composition of leaves of essential oils of *Juniperus phoenicea* from Morocco

Peak	Constituents	*RT (min)	**IK	Air (%)	***Mass range (m/z)
1	piperitone	26.96	1548	1.56	(152),82,110,39,41,27,95,137,109,54,152
2	γ-terpinene	27.01	988	1.28	(136),93,91,121,77,92,79,43,41,105
3	Trans-pinocarveol	28.80	1321	1.23	(152),92,91,70,55,41,83,79,134,69,119
4	linalool	29.42	1072	2.54	(136),93,41,69,39,91,77,79,27,92,53
5	caryophyllene oxide	31.81	1506	0.45	(220),43,41,79,93,91,95,69,55,67,81
6	α-pinene	32.49	938	49.15	(136),93,91,136,121,77,92,79,43,41,105
7	myrcene	36.08	948	5.24	(136),41,93,69,39,27,53,79,77,67,91
8	borneol	39.17	1128	0.36	(154),95,41,110,93,55,67,139,121,96,69
9	ρ-cymene	41.08	1032	1.10	(134),119,134,91,120,117,41,77,39,65,115
10	3-carene	42.59	1004	1.05	(136),93,91,79,77,92,121,80,136,94,105
11	α-phellandrene	43.82	954	7.39	(136),93,77,91,136,79,94,41,80,92,39
12	α-terpineol	47.09	1133	1.02	(154),59,93,121,136,81,43,68,95,67,41
13	γ-cardinene	49.07	1430	1.01	(204),161,189,204,105,91,119,133,27,55
14	β-caryophyllene	51.01	1984	0.98	(204),93,133,91,41;79,69,105,107,120,77
15	β-pinene	52.72	924	3.58	(136),93,91,136,121,77,92,79,43,41,105
16	bornyl acetate	54.15	1267	0.53	(196), 95,43,93,436,121,41,80,55,108,69
17	terpinolene	55.01	1042	0.91	(136),93,121,91,136,79,77,105,39,41,107
18	germacrene B	57.25	1572	0.65	(204),121,93,41,107,67,79,81,105,91,119
19	germacrene D	61.12	1505	0.68	(204),161,105,91,41,119,79,81;93,77,27
20	verbenol	63.18	1126	0.31	(125),109,41,94,81,39,69,55,91,43,57
21	camphene	68.75	933	0.28	(136),93,79,91,77,41,121,80,94,107,39
22	tricyclene	79.03	719	0.21	(136),93,91,79,41,39,77,121,136,27
23	β-eudesmol	85.42	1583	0.36	(222),59,149,43,41,108,93,79,81,67,164
Monoterpene hydrocarbons				70.19	
Oxygen monoterpene hydrocarbons				7.38	
Sesquiterpenes				3.32	
Oxygen Sesquiterpenes				0.45	
Others				0.53	
Total Identified Compounds				81.87	
Yields (%)				1.62	

*RT: Retention time obtained by chromatogram (Fig1)
**KI: Kovats Index was determined by GC-FID on a CP-Sil 5 HP column
***Mass range (m/z) was determined by mass spectrometry (PlarisQ)

Table II: Comparisons of the total oil and yield of leaves of essential oils of *Juniperus phoenicea* analyzed in other countries

Extraits	Portugal (Robert *et al.*, 1996)		Spain (Robert *et al.*, 1996)		Greece (Robert *et al.*, 1996)		Egypt (Elsawi *et al.*, 2007)		Morocco	
Juniperus	Total oil (%)	Yield (%)	Total oil (%)	Yield (%)	Total oil (%)	Yield (%)	Total oil (%)	Yield (%)	Total oil (%)	Yield (%)
phoenicea	98.3	0.41	99	0.66	88	0.58	99.16	1.96	81.87	1.62

Boulmane (Morocco) was 1.62%. It is relatively higher than other plants industrially exploited as a source of essential oils: Thymue (1%) (Imelouane *et al*., 2009a & b), lavender (0.8-2.8%), menthe (0.5-1%), néroli (0.5-1%) and Laurel (0.1-0.35%) (Edward *et al.*, 1987), Artemisia (0.65%) (Akrout *et al.*, 2001) and Tetraclinis (0.22%) (Bourkhiss *et al.*, 2007).

The chemical compositions revealed that this leaves had compositions similar to those of other *J. phoenicea* essential oils analyzed in USA by Robert *et al.* (1996), which the major component was α-pinene. Contrary it's different to the composition of essential oil of leaves of *Lavandula dentate* study in Morocco, which the major component were 1, 8 cineol (41.28%) and sabinene (13.69%) (Imeloane *et al.*, 2009a & b). Intensive research on the chemical characteristics has been conducted on this species (LeBreton, 1983; Afifi *et al.*, 1992; San Feliciano *et al.*, 1993; Robert *et al.,* 1996; Adams, 2001). The leaves essential oil of *J. phoenicea* has been reported in varying detail (Banthrope *et al.*, 1973).

In this study the yield and total oil composition of essential oils of *J. phoenicea* collected from region of Boulmane (Morocco), where 1.62% and 81.87%. The yield of essential oils of leaves of *J. phoenicea* is relatively higher than other plants study in Spain; Portugal and Greece. Contrary to the yield of essential oils of leaves of *J. phoenicea* study in Egypt, which is high level (Table II).

The essential oil content shows variations in plants of different geographical origin and also in different part of the tree: Robert *et al.* (1996), studied the composition of *J. phoenicea* oil collected from the Portugal, Spain and Greece, they reported that the yields and the total oil obtained were (0.41% & 98.3%), (0.66% & 99%) and (0.58% & 88%), respectively and the composition is characterized by a high content of α-pinene (34.1%, 53.5% & 41.8%), β-phellandrene (19.2%, 5.9% & 3.5%) and β-caryophyllene (0.22%, 1.0% & 0.5%). In our previous studies on the chemistry of Egyptian *J. phoenicea* (El-sawi *et al.*, 2007), considerable differences were observed in the essential oil composition between leaves and berries: α-

pinene (38.22% & 39.30%), (α -cedrol 31.23% & sabinene 24.29%), respectively. Furthermore the essential oils, obtained from flower, leaves and stems from basil (*Ocimum basilicum* L.) from Mersin province (Bu¨yu¨keceli-Gu¨ lnar) in Turkey contained: estragole (58.26%, 52.60% & 15.91%), limonene (19.41%, 13.64% & 2.40%) and p-cymene (0.38%, 2.32% & 2.40%).

In our previous studies on the chemistry of Uruguay (Lorenzo *et al.*, 2002), considerable differences were observed in the essential oil composition between *Mentha rotundifolia* and *M. pulegium*: Piperitenone (80.8%) and Pulegone (73.4%) and the total constituents identified is 93.5% and 99.3%, respectively.

Antibacterial activity: In the last few years, there has been target interest in biologically active compounds, isolated from plant species for the elimination of pathogenic micro-organisms, because of the resistance that micro-organisms have built against antibiotics (Essawi *et al.*, 2000), because they are ecologically safe compounds (Lee *et al.*, 2005).

Results obtained in the antibacterial study of the essential oils are shown on Table III. With the agar disc diffusion assay, oils were found to be active *E. coli, Staph. aureus*, *Staph. intermedius* and *K. pneumonia* at a minimal inhibitory concentration (MIC) of 0.02, 0.10, 0.11 and 0.18 mg/mL. Against *P. aeruginosa, B. subtilis and S. mutans* the oil from the leaves was found to be more active; the oils showed MIC values of 0.22, 0.32 and 0.40 mg/mL, respectively. The data indicated that *E. coli* were the most sensitive strain tested to the oil of *J. phoenicea* with the strongest inhibition zone (34 mm). The *Staph. aureus Staph. intermedius* and *K. pneumonia*, were found to be more sensitive among bacteria with inhibition zone of 24, 18 and 14 mm, respectively. Modest activities were observed against *P. aeruginosa, B. subtilis* and *S. mutans* with inhibition zones of 10, 10 and 8 mm.

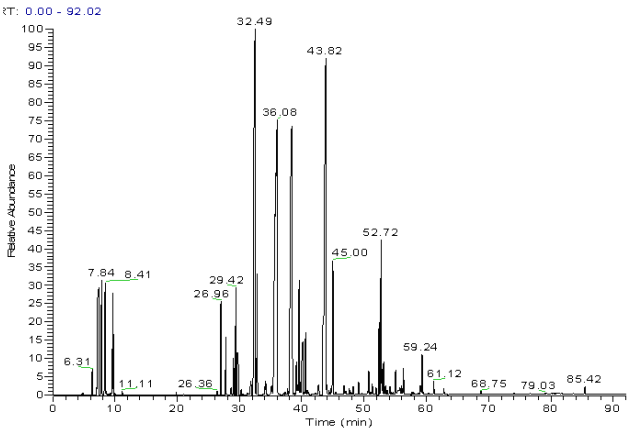
The major components of this oil, α-pinene, has been known to exhibit antimicrobial activity against the bacterial strains (*E. coli, Staph. aureus, Micrococcus luteus, B. subtilis*) (Bourkhiss *et al.,* 2007). Essential oils rich in α-pinene demonstrated potential antibacterial activity (Hajji *et al.,* 1993; Tantaoui-Elaraki *et al.*, 1993). Monoterpenes hydrocarbons, terpinenes, have also shown antimicrobial properties that appear to have strong to moderate antibacterial activity against Gram positive bacteria (Oyedeji & Afolayan, 2005). The bridged bicyclic monoterpenes *α*-pinene and *β*-pinene showed considerable biological activity (Delaquis *et al.*, 2001; Kim *et al.*, 2003; Józef *et al.*, 2006). The antimicrobial activities revealed that this leaves had similar to those of other *J.* essential oils analyzed by Angioni *et al*. (2003), which the major component was α-pinene (86%). Intensive research has been conducted on this antibacterial activity (Martin *et al*., 2000; Aligiannis *et al.*, 2001). The antimicrobial activities have been mainly explained through C_{10} and C_{15} terpenes with aromatic rings and phenolic hydroxyl groups able to form hydrogen bonds with active sites of the target enzymes,

Table III: Antibacterial activity of leaves essential oils of *Juniperus phoenicea* from Morroco

Microorganisms	Disc diffusion assay (inhibition zone mm)	MIC (mg/mL)
Escherichia coli	34	0.02
Staphylococcus aureus	24	0.10
Staphylococcus intermedius	18	0.11
Klebsiella pneumonia	14	0.18
Pseudomonas aeruginosa	10	0.22
Bacillus subtilis	10	0.32
Streptococcus mutans	8	0.40

Disc diameter 6mm average of two consecutive trials
MIC: Minimal Inhibitory Concentration, concentration range: 0.02-0.40 mg mL^{-1}

Fig. 1: Chromatogram of *Juniperus phoenicea*



although other active terpenes, as well as alcohols, aldehydes and esters can contribute to the overall antimicrobial effect of essential oils (Belletti *et al.,* 2004). On the other hand, enantiomers of α-pinene, β-pinene, limonene and linalool have a strong antibacterial activity (Magiatis *et al.,* 1999).

CONCLUSION

The chemical analyses, by GC/MS, GC-FID allowed identification of ~81.87% of the total volatile products for *J. phoenicea* and 23 volatile compounds. A major constituent in aerial parts was α-pinene (49.15%) and the yield of essential oils was 1.62%. This yield of the plants essential oil that has been studied was important. These extracts reveal *in vitro* antibacterial activity on the studied bacterial, confirmed by MIC ranging from 0.02 to 0.40 mg per mL. Antibacterial activities of these essential oils were due to abundance of the α-pinene and overall chemical constituents of this oil. The antibacterial activity besides several biological activities can be employed in place of costly antibiotics for effective control of food borne pathogens.

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